FERMENTATION PROCESS FOR THE PREPARATION OF PRAVASTATIN

Field of the Invention

The field of invention relates to a fermentation process for the preparation of substantially pure pravastatin. The process provides a method of producing pravastatin by microbial hydroxylation of compactin (ML-236B) by maintaining a concentration of compactin at not less than 300 μg /ml during the process. The process produces substantially pure pravastatin with reduced related impurities. The invention also relates to pharmaceutical compositions that include the substantially pure pravastatin.

Background of the Invention

Hypercholesterolemia or elevated plasma cholesterol level has long been recognized as a major risk factor for atherosclerotic disease, and specifically for coronary heart disease. The biosynthesis of cholesterol is a major contributing factor to hypercholesterolemia. In the rate determining step of the biosynthesis of cholesterol, HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate. It was expected that plasma cholesterol could be reduced as a result of inhibition of HMG-CoA reductase because more than 70% of the total input of body cholesterol is derived from de novo synthesis in humans.

Pravastatin, simvastatin, lovastatin, mevastatin, atorvastatin, fluvastatin, cerivastatin and derivatives and analogues thereof are known as HMG-CoA reductase inhibitors and are used as antihypercholesterolemic agents.

During the past two decades, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) has been extensively studied. In 1975, compactin (ML-236B), a potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was discovered in the culture broth of *Pencillium citrinum*. After a thorough screening of hundreds of microbial products, as well as, chemically or biologically modified derivatives of ML-236B, pravastatin sodium was chosen as a candidate for development. Pravastatin sodium was chosen because of its stronger and more tissue-selective activity than the prototype compound.

Presently, the most economically feasible process for making pravastatin is by the microbial hydroxylation of compactin at C-6 position. Microbial hydroxylation of compactin to pravastatin can be accomplished to various extents with molds belonging to different genera, such as *Mucor Rhizopus, Syncephalastrum, Cunninghamella, Mortierella* and with filamentous bacteria belonging to different genera, such as *Nocardia, Actinomadura, Streptomyces*, as described in various patents [U.S. Patent Nos. 5,179,013; 4,448,979; 4,346,227; 4,537,859; U.S. Patent Applications US2002/0081675A1; US2001/0026934A1; Japanese Patent No. 58-10572; and European Patent No. 0605230].

The most common problem encountered in the commercial production of pravastatin is that most microorganisms are not able to tolerate the compactin substrate fed even at low concentrations due to its cytotoxic effect. (Biotechnol. Bioeng., 42:815-820, 1993]. Taking into account the efficient manufacture of the active ingredient on an industrial scale, it is important to have a strain that is able to tolerate high concentrations of compactin, as well as, pravastatin.

Furthermore, the purity of the active ingredient is an important factor for the manufacturing of a safe and effective pharmaceutical product. Several structurally related products are sometimes produced during the fermentation processes, and often only one product is desired from a process, thereby requiring a method to control the ratio of these different analogues. A commercial process typically requires either exclusive or predominant production of one product. While the nature of polyketide synthase permits controlled biosynthesis of a single chemical entity, a significant number of polyketide synthases generate related products (J. Ind. Microbiol. Biotechnol., 27; 368-377, 2001). However, the known methods of producing pravastatin are ill-suited for controlling the formation of these analogues.

Summary of the Invention

In one general aspect there is provided a process for producing substantially pure pravastatin. The process includes culturing microorganisms under conditions capable of converting compactin to pravastatin by maintaining the concentration of compactin at a level of not less than about 300 μg /mL during the process.

The process is accomplished through the use of fermentation techniques known in the art, for example, the repeated fed-batch culture technique. The process may include

periodically adding quantities of compactin in the culture broth during the fermentation to maintain the concentration of compactin at not less than about 300 µg /mL during the process.

In another embodiment, the concentration of compactin is maintained at a level within the range of about 300-900 µg /mL. This embodiment may result in an about 14-fold decrease in the amount of Impurity B of Formula III and an about 7-fold decrease in the amount of the compound of Formula IV.

The compactin used in this process may be in the form of a solution. The compactin solution may include soluble salts of compactin, for example, the sodium salt of compactin. The compactin may be one or any of at least substantially purified compactin, semi-purified compactin and an intermediate compound produced during the synthesis of compactin.

The microorganisms may be any species of the genus *Streptomyces*. In one embodiment, the microorganism may be *Streptomyces carbophilus*. In other embodiments, the microorganism may be a *Streptomyces carbophilus* strain, variant or a mutant thereof.

The conditions capable of converting compactin to pravastatin include the fermentation production medium containing glucose at a concentration level of about 15-23 (g/L), Soya bean meal at a concentration level of about 25-38 (g/L), cottonseed meal at a concentration level of about 2-4 (g/L), corn steep liquor at a concentration level of about 5-8 (g/L), sodium chloride at a concentration level of about 5-6 (g/L) and calcium carbonate at a concentration level of about 2-3 (g/L).

The conditions capable of converting compactin to pravastatin may also include maintaining the temperature of the production medium at about 18 °C to about 50°C. In another embodiment, the temperature is maintained at about 25 °C to about 30 °C.

The conditions capable of converting compactin to pravastatin may also include the maintenance of the pH of the production medium from about 5 to about 10. In another embodiment, the pH is maintained from about 6 to about 8.5. In yet another embodiment, the pH may be maintained from about 7.3 to about 8.0. The conditions capable of

converting compactin to pravastatin may also include agitation at about 100 to about 600 rpm. In one embodiment, the agitation may be at about 100 to about 350 rpm.

In one embodiment of the process, the percentage conversion of compactin to pravastatin is at least about 50% w/w as determined by HPLC. The percentage conversion may be at least about 65 to about 75% w/w, or at least about 70% w/w as determined by HPLC.

In another general aspect there is provided substantially pure pravastatin containing not more than about 0.12% w/w of Impurity B and not more than about 0.6% w/w of 3"-hydroxy-pravastatin.

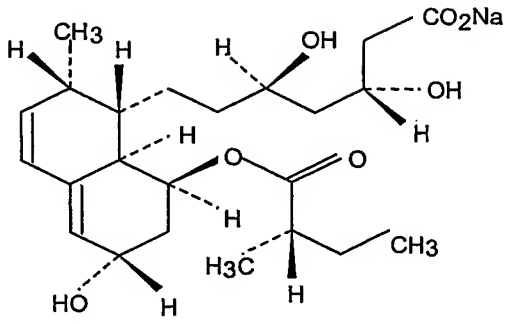
In yet another general aspect there is provided a pharmaceutical composition that includes substantially pure pravastatin containing not more than about 0.12% w/w of Impurity B and not more than about 0.6% w/w of 3"-hydroxy-pravastatin, and pharmaceutically acceptable excipients.

In another general aspect there is provided a method of treating hypercholesterolemia. The method includes administering to a patient in need of treatment for hypercholesterolemia a pharmaceutical composition that includes substantially pure pravastatin containing not more than about 0.12% w/w of Impurity B and not more than about 0.6% w/w of 3"-hydroxy-pravastatin, and other pharmaceutically acceptable excipients.

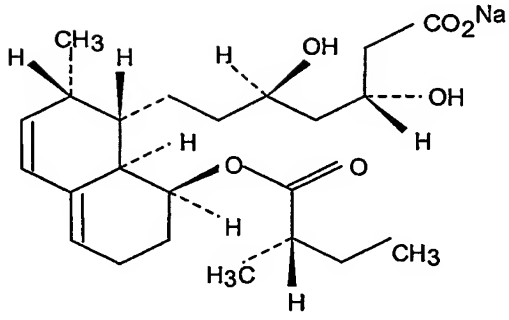
The details of one or more embodiments of the inventions are set forth in the description below. Other features, objects and advantages of the invention will be apparent from the description.

Detailed Description of the Invention

The present invention provides an efficient process for the preparation of substantially pure pravastatin of Formula I by microbial hydroxylation of compactin of Formula II. By maintaining the concentration of compactin at not less than 300 μg /ml throughout the process, the quantities of Impurity B and 3"-(S)-hydroxy pravastatin, of Formula III and IV respectively, are substantially reduced.



FORMULA I



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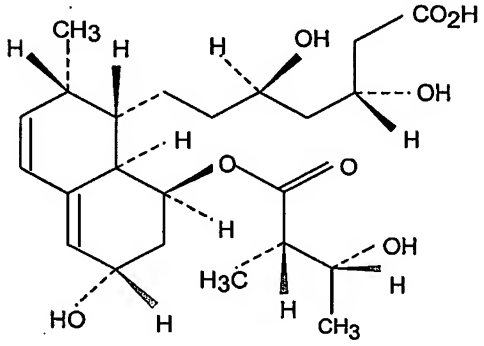
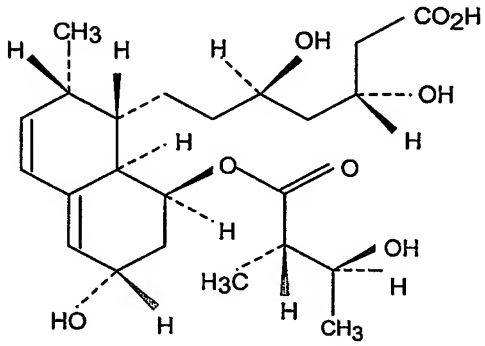


FORMULA III



FORMULA IV

Compactin is provided and contacted with whole cells of *Streptomyces* sp. under the conditions in which the microorganism converts compactin to pravastatin. The process may be carried out subsequent to or during the growth of the microorganism to be employed. The compactin substrate is placed in contact with the microorganisms and is converted to pravastatin. Compactin may be provided in the form of a solution comprising the sodium salt of compactin, and may be the purified, crude or intermediate stage of compactin.

The term "substantially pure pravastatin" as used herein is defined as pravastatin or a pharmaceutically acceptable salt thereof having a purity of not less than 99.3% w/w wherein the impurity B and 3"-hydroxypravastatin are not present at not more than about 0.12% w/w of Impurity B and not more than about 0.6% w/w of 3"-hydroxy-pravastatin as determined by HPLC.

The conversion of compactin to pravastatin can be done using fermentation techniques known in the art; for example, those of the types useful for large-scale

industrial fermentation process, such as batch, fed-batch or continuous culture techniques. For example, agitated liquid submerged culture techniques can be used.

The growth of the microorganism may be achieved through the use of an appropriate medium containing nutrients, such as carbon, nitrogen sources and trace elements, which are added to the culture medium. Suitable assimilable carbon sources include one or more of glucose, glycerol, maltose, dextrin, starch, sucrose etc. Suitable nitrogen sources include one or more of soybean meal, peptones, cottonseed meal, corn steep liquor, meat extract, yeast extract, ammonium sulfate, ammonium nitrate etc. Suitable inorganic salts include one or more of sodium chloride, phosphates, calcium carbonate etc.

Suitable seed medium may include glucose at concentrations of about 16-25 (g/L), Soya bean meal at concentrations of about 4-6 (g/L), peptone at concentrations of about 4-6 (g/L), potassium phosphate at concentrations of about 0.08-0.13 (g/L) and calcium carbonate at concentrations of about 4-6 (g/L). For example, the seed medium used can comprise glucose at concentrations of about 20 (g/L), Soya bean meal at concentrations of about 5(g/L), peptone at concentrations of about 5 (g/L), potassium phosphate at concentrations of about 0.1 (g/L) and calcium carbonate at concentrations of about 5 (g/L).

Suitable production medium may include glucose at concentrations of about 15-23 (g/L), Soya bean meal at concentrations of about 25-38 (g/L), cottonseed meal at concentrations of about 2-4 (g/L), corn steep liquor at concentrations of about 5-8 (g/L), sodium chloride at concentrations of about 5-6 (g/L) and calcium carbonate at concentrations of about 2-3 (g/L). For example, the production medium used may include glucose at concentrations of about 18 (g/L), Soya bean meal at concentrations of about 30 (g/L), cottonseed meal at concentrations of about 3 (g/L), corn steep liquor at concentrations of about 6 (g/L), sodium chloride at concentrations of about 6 (g/L) and calcium carbonate at concentrations of about 2.4 (g/L).

The incubation temperature can be about 18°C to about 50°C, for example, about 25°C to about 30°C or for example about 26°C to about 28°C. Suitable pH of the culture broth may range from about 5 to about 10, for example, about 6.0 to 8.5, or for example, about 7.3 to about 8.0. The process may be carried out under aerobic conditions, such as by means of aeration and/or agitation. The fermentation broth may be agitated at about

100 to 600 rpm, for example, at about 100 to about 350 rpm or for example, at about 150 to 300 rpm.

The amount of compactin at the onset of the process can be, for example, in the range from about 300 μg/mL to about 1800 μg/mL of the culture medium, or for example, in the range of about 300 μg/mL to about 900 μg/mL.

The resulting substantially pure pravastatin recovered from processes described herein contains not more than about 0.12% w/w of Impurity B and not more than about 0.6% w/w of 3"-hydroxy-pravastatin as determined by HPLC.

The substantially pure pravastatin can be formulated into a dosage form with other pharmaceutically acceptable excipients. Optional excipients include, but are not limited to, one or more of colorants, diluents, lubricants, binders, disintergrants, and glidants. Suitable dosage forms include one or more of tablets, capsules, dispersions, and liquids.

Any percentage of conversion of compactin to pravastatin can be practiced according to processes described herein, for example at least about 50% w/w, or for example, at least about 60%w/w, or for example, about 65-75% w/w.

While the present invention has been described in terms of its specific embodiments, certain modifications and equivalents will be apparent to those skilled in the art and are intended to be included within the scope of the present invention.

Example 1: Bioconversion of Compactin to Pravastatin – Uncontrolled Compactin Concentration Level

This example illustrates the bioconversion of compactin to pravastatin by *Streptomyces carbophilus*. The seed medium was inoculated with a slant culture at 28°C for 2 days. 400 ml of mature seed culture was added to a 20 L production fermenter.

The components of the seed and production medium employed in these examples are as follows:

A seed medium containing glucose 20 (g/L), Soya bean meal 5 (g/L), peptone 5 (g/L), potassium phosphate 0.1 (g/L) and calcium carbonate 5 (g/L) was inoculated with spores of *Streptomyces* sp. from a slant culture, and cultured at 500 rpm, 28°C for 2 days, to give a seed culture. 400 ml of this seed culture was added to 20-L production fermenter

containing glucose 18 (g/L), soya bean meal 30, (g/L) cottonseed meal 3 (g/L), corn steep liquor 6 (g/L), sodium chloride 6 (g/L) and calcium carbonate 2.4 (g/L).

The medium was pre-sterilized at 121°C for 30 minutes. After the growth of the microorganism, an initial shot of compactin was added to the fermenter to a level of 500 μg/ml and further cultured. Compactin was further added when the concentration of compactin level was less than 50 μg/ml. The rate of compactin bioconversion was monitored by HPLC. The percentage of conversion was calculated on the basis of compactin added and pravastatin produced. Concentrations (w/w) of Impurity B and 3"S hydroxy pravastatin were also determined by HPLC.

The levels of impurity B and 3"S hydroxy pravastatin present were in the range of 0.9 to 1.27 % w/w (Figure 1) and 1.84 to 2.96 % w/w (Figure 2), respectively, as determined by HPLC.

Example 2: Bioconversion of Compactin to Pravastatin – Controlled Compactin Concentration Level

The procedure of Example 1 was followed but the concentration of compactin was maintained between 300-900 μg/ml throughout the batch as determined by HPLC. Compactin was added when the concentration reached the lowest value in the range.

The concentration levels of impurity B and 3"S hydroxy pravastatin were in the range of 0.08 to 0.12% w/w and 0.4 to 0.6% w/w respectively. These values represent about 14-fold and 7-fold lower impurity levels, respectively, as compared to the pravastatin obtained in Example 1 (Figures 1 and 2).

The resultant product of Example 2 was purified using an industrial scale recovery process detailed in PCT Patent Application WO 01/44144. The purity of pravastatin obtained is about 99.4% w/w as determined by HPLC.

Example 3: Kinetics of Compactin Bioconversion

This example illustrates the kinetics of the conversion of compactin to pravastatin. The medium and culture conditions employed were the same as in Example 1.

After 2 days of microorganism growth, presterilized compactin solution was added to the fermenter to a level of 300-900 μg/ml and further cultured. Compactin was further

added so as to maintain the compactin concentration level at the prescribed range. The rate of compactin bioconversion at different time intervals was monitored for the next 6 days by HPLC. Percentage conversion was calculated on the basis of compactin charged and pravastatin produced. The concentration levels of Impurity B and 3"S hydroxy pravastatin were also determined by HPLC. The data is provided in Figure 1 and Figure 2 respectively.

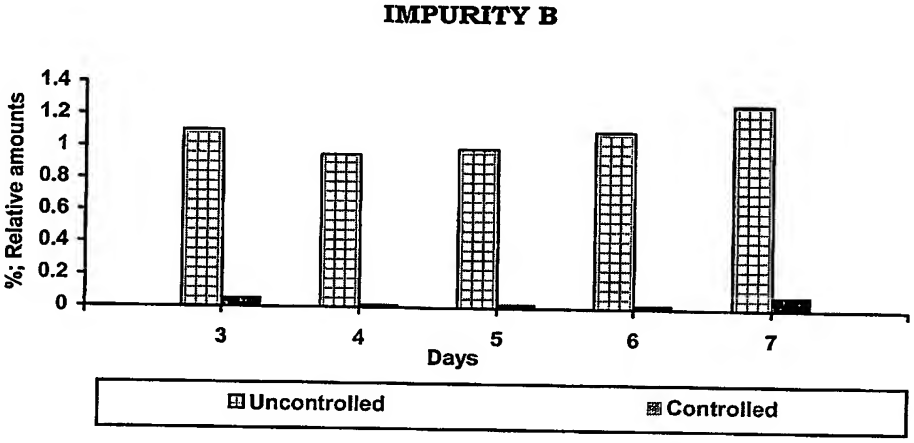


FIGURE 1

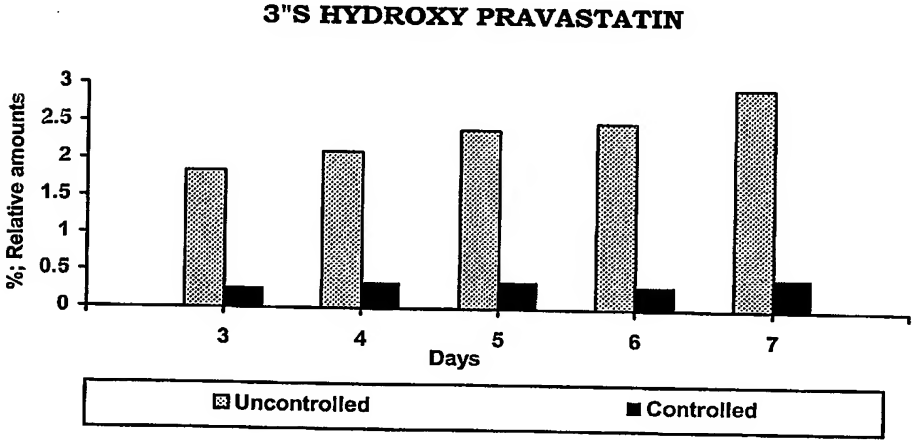


FIGURE 2

The results indicate that pravastatin is obtained with very low quantities of related compounds, Impurity B and 3"S hydroxy pravastatin, when the concentration of compactin was maintained between 300 and 900 μg/mL.

While several particular forms of the invention have been illustrated and described, it will be apparent that various modifications and combinations of the invention detailed in

the text can be made without departing from the spirit and scope of the invention. Further, it is contemplated that any single feature or any combination of optional features of the inventive variations described herein may be specifically excluded from the claimed invention and be so described as a negative limitation.